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INVESTIGATIVE REPORT

Serum Reactivity Against Herpes Simplex Virus Type 1 UL48 Protein in Behçet's Disease Patients and a Behçet's Disease-like Mouse Model

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Herpes simplex virus (HSV) infection is a possible pathogenic factor in Behçet's disease (BD). Using proteomics analysis, this study detected a target HSV protein. Serum IgA and IgG reactivities against the identified protein were evaluated in patients with BD and in BD-like mice. A total of 4 protein bands generated by immunoprecipitation were analysed by proteomics, and HSV UL48 was commonly found in both IgA- and IgG-reactive protein bands. Compared with controls, patients with BD and BD-like mice exhibited higher titres of IgA reacting with recombinant HSV UL48 protein. Further proteomics analysis revealed that human heat shock cognate 71 kDa protein (Hsc71) is a cross-reacting target antigen against anti-HSV UL48 antibody. In addition, our data demonstrated a very strong association between serum IgG reactivity against recombinant human Hsc71 and recombinant HSV UL48 in patients with BD. We suggest that HSV infection and impaired human Hsc71 activity may be associated with the activation of autoreactive lymphocytes. ***Key words: Behçet's disease; herpes simplex virus; UL48; Behçet's disease-like mouse model; proteomics; heat shock cognate 71 kDa protein*****.**

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Behçet's disease (BD) is an autoimmune or autoinflammatory disorder that causes chronic multisystemic vasculitis (1, 2). However, the exact pathogenesis of vascular inflammatory reactions in BD is unknown. Although BD is not considered a classical infectious disease, microbial infection and associated immune dysregulation have been suggested as a major triggering factor in the pathogenesis of the disorder (2, 8). Among the suggested infectious agents, *Streptococcus sanguinis* and herpes simplex virus (HSV) are thought to play a crucial role in inducing dysregulated immune reaction in this disease (5, 8).

Bacterial heat shock proteins (Hsp) have structural similarities with human Hsps and can induce strong immunological reactions in humans (9–12). To date, several studies have demonstrated that various infectious organisms can induce multiple cross-reactions with human proteins in genetically predisposed individuals, resulting in the development of organism-specific autoimmune diseases (9–12). Our study group also previously demonstrated that serum IgA antibody in patients with BD significantly reacts with human heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1 in endothelial cells and chaperonin GroEL of *S. sanguinis* (9, 13). In the current study patients with BD exhibited serum IgA cross-reactivity against human hnRNP A2/B1 and *S. sanguinis* GroEL, and marked serum IgA reactivity against specific homologous epitope regions of these proteins (9, 13).

Following the discovery of evidence of HSV infection in the saliva and the ulcerative lesions of patients with BD, the pathogenetic role of HSV in BD has been investigated (4, 7, 8). In addition, a BD-like mouse model has been developed by inoculating HSV into the earlobes of mice, triggering HSV-induced dysregulated immune responses and BD-like multi-organ symptoms (14, 15).

The aim of the present study was to identify anti-HSV antigens that react with serum IgA and/or IgG in patients with BD. As described in previous studies (9, 13), target HSV proteins were screened using immunoprecipitation, and liquid chromatography-matrix-assisted laser desorption/ionization-tandem time-of-flight (LC-MALDI-TOF/TOF) was performed to analyse the detected protein bands. The serum reactivity of the recombinant HSV target protein in patients with BD and a HSV-induced BD-like mouse model were then investigated, using enzyme-linked immunosorbent assay (ELISA) and Western blot analysis.

MATERIALS AND METHODS

Patients

A total of 60 patients with BD (19 males, 41 females; mean age 40.9 ± 10.8 years; age range 19–63 years) were included in this study, in whom BD was diagnosed according to the diagnostic criteria set by the International Study Group for BD (16). The following symptoms were observed, in order of most to least frequent: recurrent oral ulcers ($n = 60$, 100%), skin

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lesions ($n = 55$, 91.7%), genital ulcers ($n = 50$, 83.3%), articular involvement ($n = 30$, 50%), ocular involvement ($n = 17$, 28.3%), gastrointestinal lesions ($n = 4$, 6.7%), vascular involvement ($n = 1$, 1.7%), and epididymitis ($n = 1$, 1.7%). Positivity for HLA-B51 was noted in 17 patients (28.3%). Serum samples were obtained from all of the patients with BD when they were in a clinically active disease state, presenting at least 2 major criteria. Samples were stored at –70°C. Purchased sera from 60 healthy donors (Innovative Research Inc., Novi, MI, USA) were included as controls. This study was approved by the Institutional Review Board of Severance Hospital, Yonsei University College of Medicine, Seoul, Korea.

Behçet's disease-like mouse model

A BD-like mouse model was developed by inoculating HSV type 1 grown in Vero cells in 4–5-week-old male Institute of Cancer Research (ICR) mice (14). Briefly, the earlobes of experimental mice were scratched with a needle and they were inoculated twice with the virus, with a 10-day interval. The infected mice were observed for 16 weeks after the final inoculation. HSV-inoculated mice presenting at least 2 BD-like symptoms were used as a BD-like mouse model ($n = 5$) (14, 15). Both uninfected ICR mice ($n = 2$) and HSV-inoculated, but asymptomatic, mice ($n = 2$) were included as controls. The animal study was approved by the animal care committee of the Ajou University School of Medicine (Suwon, Korea).

Immunoprecipitation and liquid chromatography-matrix-assisted laser desorption/ionization-tandem time-of-flight analysis (see Appendix S1[1])

Construction of expression vectors and bacterial expression (see Appendix S1[1])

Western blotting using recombinant herpes simplex virus type 1 UL48 (see Appendix S1[1])

Enzyme-linked immunosorbent assay (see Appendix S1[1])

Immunohistochemical study (see Appendix S1[1])

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Statistical analysis

Values for quantitative variables are described as the median and interquartile range (IQR). Serum reactivity against recombinant HSV type 1 UL48 and recombinant human Hsc71 proteins were compared between patients with BD and healthy subjects, as well as between BD mice and control mice, using non-parametric Mann-Whitney *U* tests, χ^2 tests, Fisher's exact tests, and Spearman's rank correlation coefficients. The diagnostic accuracy of Western blotting and ELISA using recombinant HSV type 1 UL48 protein were analysed in patients with BD by the area under the receiver operating characteristic curve (AUROC) with comparisons using DeLong's method. All analyses were performed using SAS software version 9.2 (SAS Institute Inc., Cary, NC, USA). All p-values < 0.05 were considered statistically significant.

RESULTS

Immunoprecipitation and proteomics analysis

Extracts of HSV reacting with IgA and IgG antibodies, respectively, were immunoprecipitated in serum samples from 5 patients with BD (Fig. 1a, b). A total of 4 protein bands obtained by IgA ($n = 2$) and IgG ($n = 2$) immunoprecipitation were analysed by LC-MALDI-TOF/TOF, and the National Center for Biotechnology Information (NCBI) database was then searched for the DNA sequence corresponding to these 4 protein bands. After excluding data containing false positives and immunoglobulins, the viral proteins were specifically found to yield a Mascot score greater than 30 as shown in Table 1. Among the identified proteins, HSV UL48 was commonly found in both IgA- and IgG-reactive protein bands with Mascot scores of 158 and 173 and sequence coverages of 14% and 16%, respectively. Therefore, we further investigated HSV UL48 (VP16) protein in the present study.

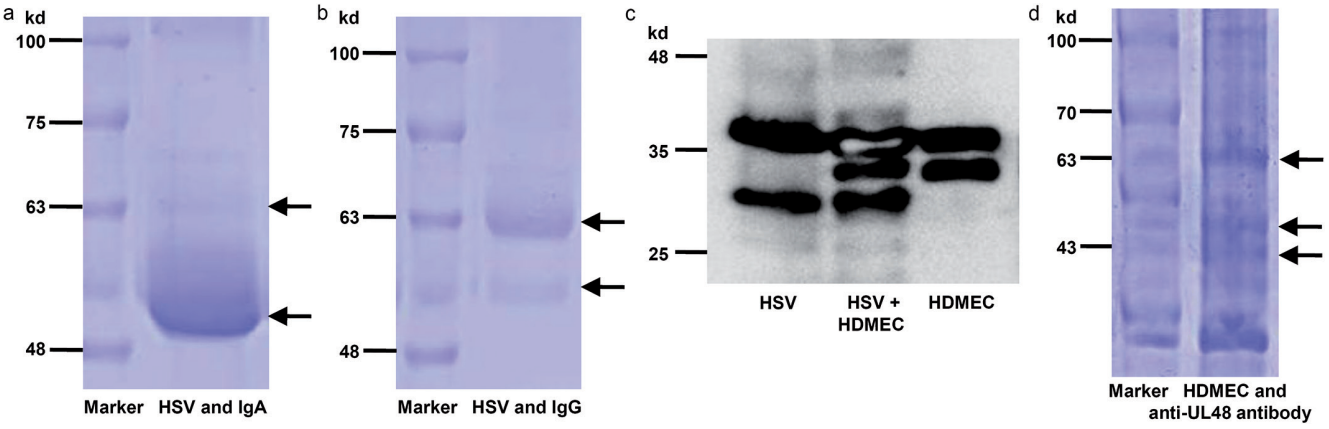

Fig. 1. Western blotting and immunoprecipitation using herpes simplex virus (HSV) and human dermal microvascular endothelial cells (HDMEC). Immunoprecipitation was performed to identify the anti-HSV (a) IgA and (b) IgG antibody-binding antigen using extracts of HSV with mixed serum samples from 5 patients with Behçet's disease (BD). A total of 4 protein bands obtained by immunoprecipitation (*arrows*) were analysed by proteomics. (c) Western blotting was performed to screen the reactive HDMEC antigens against anti-HSV UL48 antibody using HSV, HSV-infected HDMEC, and HDMEC. (d) Immunoprecipitation was subsequently performed using HDMEC antigen and anti-HSV UL48 antibody, and 3 protein bands (*arrows*) were analysed by proteomics.

Table I. *Herpes simplex virus proteins that reacted with serum immunoglobulin (Ig)A and IgG antibodies in subjects with Behçet's disease*

Identified protein	Swiss-Prot accession No.	NCBI accession No.	M_r (kDa)/p*I*	Mascot score
IgA				
UL48	ABI63509	gi | 114318835	54698/4.92	158
Alpha trans inducing factor	AAA45766	gi | 330055	53396/4.95	133
Genome polyprotein	P07564	gi | 130428	382498/8.75	69
IgG				
Glycoprotein D	ABM52980	gi | 121277019	43744/8.10	182
UL48	ABI63509	gi | 114318835	54698/4.92	173
Alpha trans inducing factor	AAA45766	gi | 330055	53396/4.95	158
Envelope glycoprotein D	P06476	gi | 138233	43767/8.89	107
Glycoprotein C-1	CAD13356	gi | 17426768	55560/7.16	54
Genome polyprotein	P14335	gi | 130494	384959/8.70	54

Serum reactivity against recombinant herpes simplex virus UL48 in patients with Behçet's disease

ELISA and Western blot analysis with recombinant HSV UL48 were performed to evaluate serum reactivity in sera from 30 patients with BD and 30 healthy controls. The median optical density (ODs) (IQR) of serum IgA reactivity against recombinant HSV UL48 obtained from ELISA were estimated as 0.35 (0.02–1.01) in patients with BD and 0.16 (0.02–0.40) in healthy controls ($p<0.001$) (Fig. 2a). Western blot analyses revealed the reactivity of serum IgA against recombinant HSV UL48 in 10 patients with BD (33.3%) and 2 healthy controls (6.7%) ($p=0.010$) (Fig. 2c). In addition, the median ODs (IQR) of serum IgG reactivity against recombinant HSV UL48 were 0.58 (0.31–1.22) in patients with BD and 0.53 (0.18–0.74) in healthy controls ($p>0.05$) (Fig. 2b). Western blot analyses revealed the reactivity of serum IgG against recombinant HSV UL48 in 18 patients with BD (60%) and 4 healthy controls (13.3%) ($p<0.001$) (Fig. 2c).

For serum IgA reactivity against recombinant HSV type 1 UL48 protein, ELISA more accurately predicted the diagnosis of BD than Western blotting (AUROC ELISA vs. Western blotting, 0.852 vs. 0.633, $p=0.003$). However, for serum IgG reactivity against recombinant HSV type 1 UL48 protein, there was no significant difference between ELISA and Western blotting in predicting the diagnosis of BD (AUROC ELISA vs. Western blotting, 0.651 vs. 0.733, $p>0.05$).

Serum reactivity against recombinant herpes simplex virus UL48 in a Behçet's disease-like mouse model

ELISA and Western blot analysis with recombinant HSV UL48 were performed to evaluate serum reactivity in sera from 5 BD-like mice, 2 uninfected ICR mice, and 2 HSV-inoculated, but asymptomatic, mice. The median ODs (IQR) of serum IgA reactivity against recombinant HSV UL48 obtained from ELISA were estimated as 0.31 (0.14–0.75) in BD-like mice and 0.10 (0.07–0.14) in uninfected ICR mice and HSV-inoculated, but asymptomatic, mice ($p=0.037$) (Fig. 3a). However, Western blot analyses revealed the reactivity of serum IgA against recombinant HSV UL48 in all 5 BD-like mice (100%), but not in the uninfected ICR mice or asymptomatic mice ($p=0.008$) (data not shown).

In addition, the mean ODs of serum IgG reactivity against recombinant HSV UL48 obtained from ELISA were 0.07 (0.04–0.11) in BD-like mice and 0.04 (0.02–0.08) in uninfected ICR mice and HSV-inoculated, but asymptomatic, mice ($p>0.05$) (Fig. 3b). Western blot analyses revealed serum IgG reactivity against recombinant HSV UL48 in only 2 of the 5 BD-like mice (40%), and in none of the uninfected ICR mice and HSV-inoculated, but asymptomatic, mice ($p>0.05$) (data not shown).

Immunohistochemical staining

Tissue samples of knee joints obtained from uninfected ICR mice and HSV-infected, but asymptomatic, mice were found to have no or scanty inflammatory cell infiltration (Fig. 4a–d), whereas marked inflammatory cell infiltration was observed in the joints from BD-like mice (Fig. 4e, f). The experimental mice of all groups exhibited mild immunoreactivity to anti-HSV UL48 antibody in the epithelium of synovial membranes. In

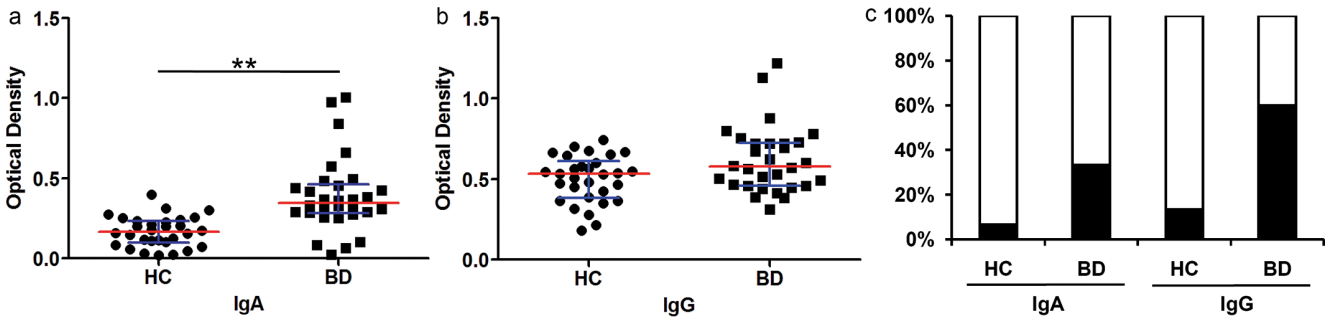

Fig. 2. Enzyme-linked immunosorbent assay (ELISA) and Western blotting using recombinant herpes simplex virus (HSV) UL48 protein. (a, b) ELISA and (c) Western blotting using recombinant HSV UL48 was performed using the sera from patients with Behçet's disease (BD) and healthy controls (HC) as a primary antibody and goat anti-human IgA and IgG antibody as a second antibody. **$p<0.01$. □: negative; ■: positive.

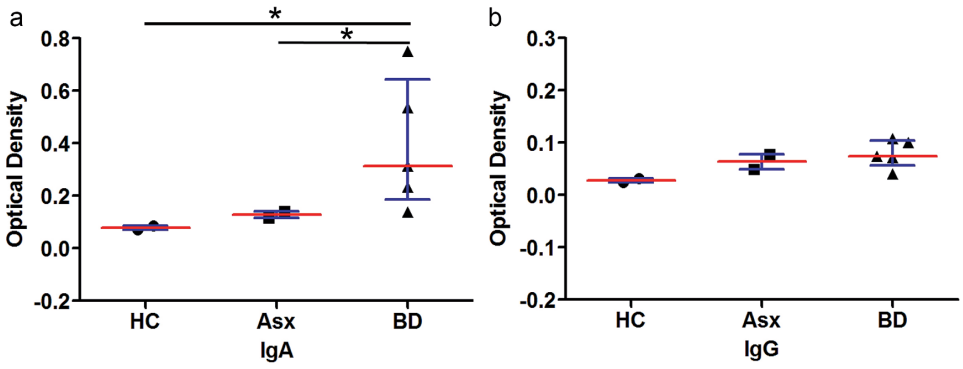

Fig. 3. Enzyme-linked immunosorbent assay (ELISA) using recombinant herpes simplex virus (HSV) UL48 protein in a Behçet's disease (BD)-like mouse model. ELISA using recombinant HSV UL48 was performed using the sera from BD-like mice, uninfected Institute of Cancer Research (ICR) mice (HC), and HSV-inoculated, but asymptomatic, mice (Asx) as a primary antibody and goat anti-mouse (a) Immunoglobulin (Ig)A and (b) IgG antibody as a second antibody. *$p<0.05$.

addition, both mononuclear cells and endothelial cells exhibited marked immunoreactivity to anti-HSV UL48 antibody in joint tissue samples from BD-like mice.

Immunoreactivity of anti-herpes simplex virus UL48 antibody against human dermal microvascular endothelial cells

In order to identify an additional target protein, which exhibited immunoreactivity to anti-HSV UL48 antibody, Western blot analysis using HSV type 1, HDMECs incubated with HSV, and HDMECs was performed, and showed that anti-HSV UL48 polyclonal antibody recognized distinct protein bands in HDMECs, compared with HSV type 1 (Fig. 1c). Immunoprecipitation was performed and 3 protein bands were analysed by proteomics as described above (Fig. 1d). After excluding data containing false positives and immunoglobulins, 8 human proteins were specifically found to yield a Mascot score larger than 30 (Table II). Among the identified proteins, Hsc71 isoform 1 was further investigated in this study.

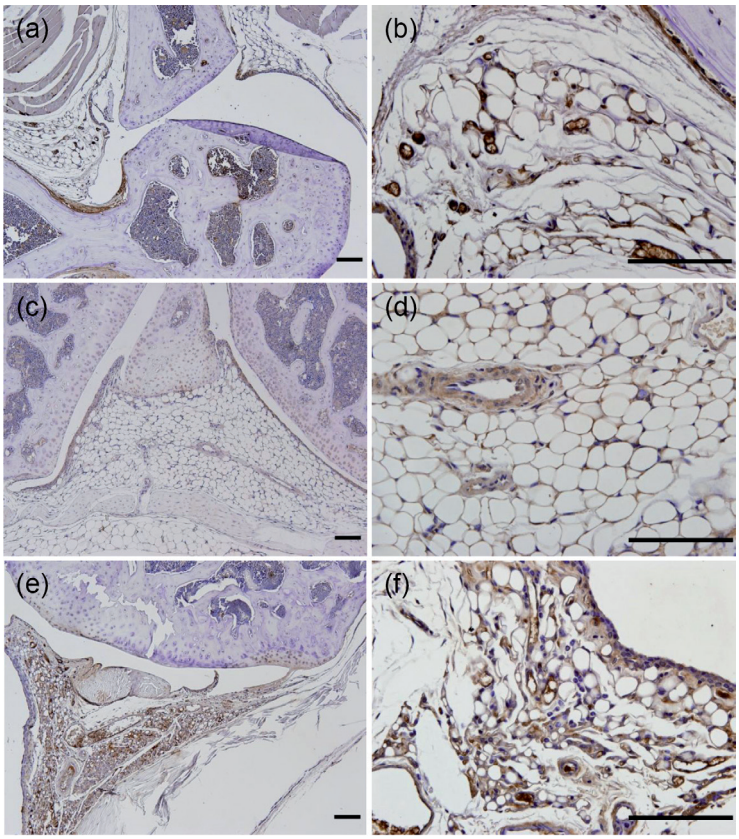

Fig. 4. Immunochemical staining of herpes simplex virus (HSV) UL48. Tissue samples of knee joints obtained from (a, b) uninfected ICR mouse, (c, d) asymptomatic mouse, and (e, f) Behçet's disease (BD)-like mice were stained with anti-HSV UL48 antibody. Mononuclear cells and endothelial cells presented marked immunoreactivity to anti-HSV UL48 antibody in tissue samples from BD-like mice. Bars = 100 µm.

Serum samples from another 30 patients with BD and 30 healthy controls were also obtained, and ELISAs with recombinant HSV UL48 protein and recombinant human Hsc71 were performed. The median ODs (IQR) of serum IgA reactivity against recombinant human Hsc71 were 0.35 (0.22–0.53) in patients with BD and 0.27 (0.18–0.35) in healthy controls ($p>0.05$) (Fig. 5a), whereas those of serum IgG reactivity against recombinant human Hsc71 were 1.19 (0.84–1.47) in patients with BD and 0.90 (0.65–1.09) in healthy controls ($p=0.015$) (Fig. 5b). In addition, the median ODs (IQR) of serum IgA reactivity against recombinant HSV UL48 were 0.67 (0.52–1.11) in patients with BD and 0.31 (0.12–1.02) in healthy controls ($p<0.0001$) (Fig. 5c), whereas those of serum IgG reactivity against recombinant HSV UL48 were 0.68 (0.27–0.95) in patients with BD and 0.65 (0.35–0.81) in healthy controls ($p>0.05$) (Fig. 5d).

ODs obtained from ELISAs against recombinant human Hsc71 were correlated with those against recombinant HSV UL48 in patients with BD: Hsc71 IgA and UL48 IgG ($r=0.402$, $p=0.027$), Hsc71 IgG and UL48 IgA ($r=-0.787$, $p<0.0001$), and Hsc71 IgG and UL48 IgG ($r=0.887$, $p<0.0001$) (Fig. 6). In ELISAs of the serum samples of healthy subjects, only Hsc71 IgA and UL48 IgA ($r=0.713$, $p<0.0001$) were correlated (Fig. 6).

Table II. *Proteins of human dermal microvascular endothelial cells that reacted with anti-herpes simplex virus UL48 protein antibody*

Identified protein	Swiss-Prot accession No.	NCBI accession No.	M_r (kDa)/p*I*	Mascot score
Keratin 1	AFA52006	gi 375314779	66197/8.15	681
Epidermal cytokeratin 2	AAC83410	gi 181402	66110/8.07	324
Vimentin	CAG28618	gi 47115317	53604/5.09	214
Keratin 6B isoform CRA_a	EAW96626	gi 119617032	60159/8.38	158
HSC71 kDa protein isoform 1	NP_006588	gi 5729877	71082/5.37	79
Alpha-tubulin	CAA25855	gi 37492	50810/5.02	73
Factor VII active site mutant ic	AAK58686	gi 28269794	77386/6.60	65
MTHSP75	AAA67526	gi 292059	74019/5.97	62

NCBI: National Center for Biotechnology Information; HSC: heat shock cognate; ic: immunoconjugate.

DISCUSSION

HSV is composed of 4 components, including a double-stranded DNA-containing electron-dense core, an icosadeltahedral capsid, a tegument, and an envelope (17). Tegument proteins have been shown to stimulate transcription of viral genes and counteract host defences to survive within an infected host (18). UL48, an abundant 65-kDa virion phosphoprotein, is one of the surrounding tegument proteins. This protein is synthesized late in infection and has a potent transcriptional activation domain on its C-terminal portion (18, 19). UL48 also modulates host innate anti-viral immune responses by antagonizing interferon (IFN)-β production by inhibiting NF-κB and blocking IFN regulatory factor (IRF)-3 (18).

In the present study, we demonstrated HSV UL48 protein as a target protein of serum anti-HSV IgA and IgG antibodies in patients with BD and a BD-like mouse model. Thirty patients with BD exhibited higher titres of IgA and IgG reacting with recombinant HSV UL48, compared with controls, by ELISA; nevertheless, statistical significance was observed only for anti-HSV UL48 IgA antibody. When we additionally collected the serum samples from another 30 patients with BD and 30 healthy controls for ELISAs, the results of serum IgA and IgG reactivity against recombinant HSV UL48 were compatible with those of the aforementioned 30 patients with BD and 30 healthy controls.

Our data also revealed marked immunostaining of anti-HSV UL48 antibody in tissue samples obtained from the knee joints of BD-like mice, compared with those from the control experimental mice. Thereafter, we further investigated cross-reacting target antigens with anti-HSV UL48 antibody, and proteomics analyses revealed that human Hsc71 also reacted with anti-HSV UL48 antibody. ODs obtained from ELISA demonstrated a very strong association ($r > 0.8$) between serum IgG reactivity against recombinant human Hsc71 and recombinant HSV UL48 in patients with BD. In addition, strong association ($0.8 > r \geq 0.6$) was found between serum IgG reactivity against recombinant human Hsc71 and serum IgA reactivity against recombinant HSV UL48 in patients with BD.

Hsc71 is a constitutively expressed form of Hsp70 and transports cytosolic proteins to lysosomes for proteasomal degradation (20, 21). The precise role of Hsp70 family and anti-Hsp70 family antibodies in the pathogenesis of autoimmune disease remains unknown (21, 22). Potent immunomodulatory properties of human Hsp70 have been reported to regulate T-cell activities, and microbial Hsp70 has also been shown to suppress host inflammatory reactions (22–24). A recent investigation suggested that Hsp70 may maintain immune homeostasis by enhancing the immunosuppressive activity of Tregs to neutralize exaggerated immune responses (22).

As Hsps are highly conserved proteins, which are widely expressed in both prokaryotes and eukaryotes with high degree of homology, common immunogenic epitopes have been found between Hsp70 family and autoantigens, as well as infectious organisms (25). Previous *in vivo* study demonstrated that the administration of both Hsp70 and antigen can induce autoimmunity (26). Anti-Hsp70 family antibodies have been detected in the sera of patients with various autoimmune disorders, including systemic lupus erythe-

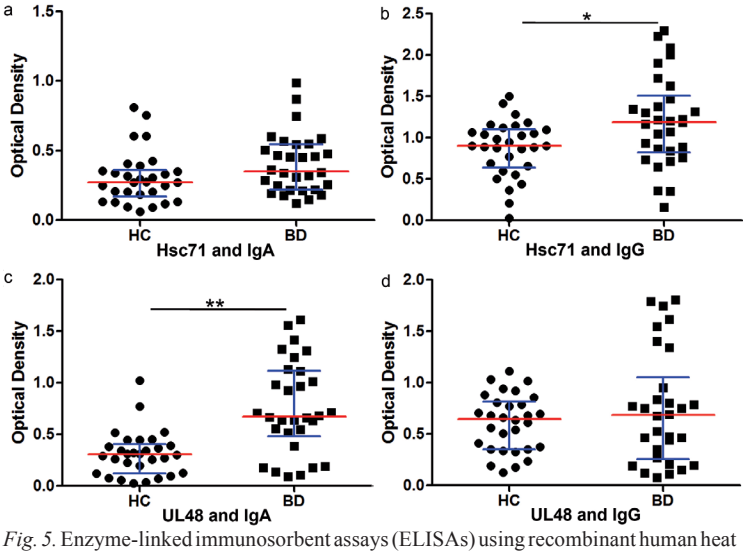

Fig. 5. Enzyme-linked immunosorbent assays (ELISAs) using recombinant human heat shock cognate 71 kDa (Hsc71) protein and recombinant herpes simplex virus (HSV) UL48 protein. ELISAs were performed using the sera from patients with Behçet's disease (BD) and healthy controls (HC) as a primary antibody and goat anti-human IgA and IgG antibody as a second antibody against (a, b) recombinant human Hsc71 HSV UL48 and (c, d) recombinant HSV UL48. *$p < 0.05$; **$p < 0.01$. Median (blue line) and interquartile range (red line).

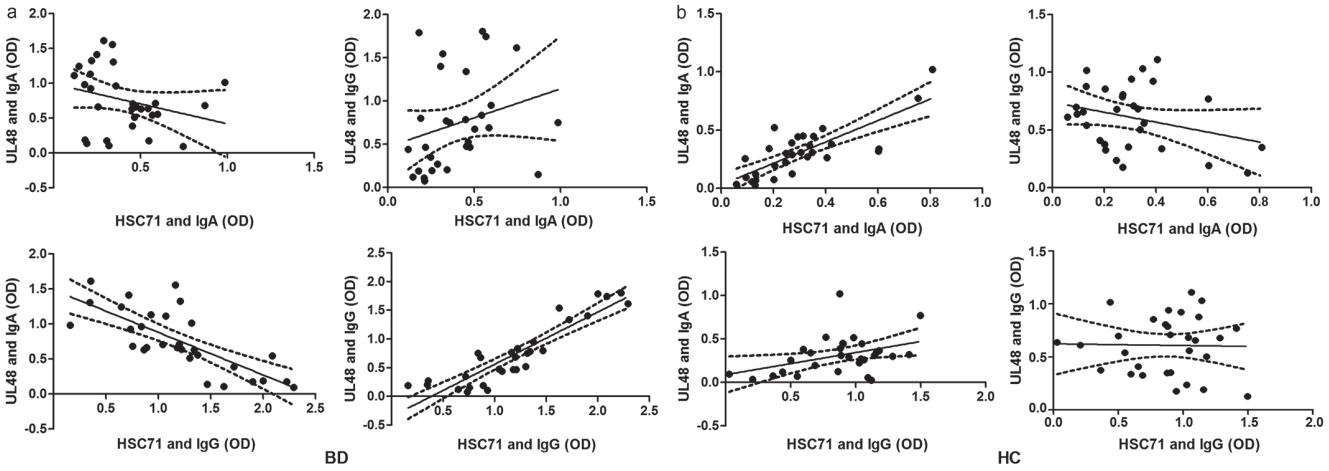

Fig. 6. Correlation of enzyme-linked immunoassay (ELISA) using recombinant proteins. Optical densities (ODs) obtained from ELISAs against recombinant human heat shock cognate 71-kDa (Hsc71) protein were significantly correlated with those against recombinant herpes simplex virus (HSV) UL48 protein in (a) patients with Behçet's disease (BD): Hsc71 IgA and UL48 IgG ($r=0.402$, $p=0.027$), Hsc71 IgG and UL48 IgA ($r=-0.787$, $p<0.0001$), and Hsc71 IgG and UL48 IgG ($r=0.887$, $p<0.0001$); (b) healthy controls (HC): Hsc71 IgA and UL48 IgA ($r=0.713$, $p<0.0001$). Trend lines were analysed by linear regression with 95% confidence intervals (95% CI) (dotted lines) using the GraphPad Prism software.

matosus, rheumatoid arthritis, mixed connective tissue disease, idiopathic thrombocytopenic purpura, and myasthenia gravis (20, 27). Paediatric patients with immune thrombocytopenic purpura, which is an autoimmune platelet-destructive disease, reportedly exhibit a high prevalence of IgG autoantibody against Hsp71 in plasma (27). As well, significantly higher titres of anti-Hsc71 antibody were detected in patients with myasthenia gravis, compared with the healthy controls (20). In addition, "therapy-responsive group" patients with myasthenia gravis exhibited significantly reduced Hsc71 antibody titres, in addition to clinical improvement (20).

In the previous report, patients with BD also exhibited significantly elevated human Hsp70 and anti-Hsp70 antibodies, and the authors suggested the crucial role of Hsp70-mediated innate and adaptive immune responses in the pathogenesis of BD by activating proinflammatory cytokine (28). In addition, the serum levels of Hsp70 were elevated in patients with BD with uveitis compared with either patients with BD without uveitis or idiopathic uveitis (29). However, there was no statistical difference among BD with uveitis, BD without uveitis, and idiopathic uveitis in the serum anti-Hsp70 antibody levels (29). Overexpressed Hsc71 inhibits host cellular antiviral responses by suppressing virus-triggered transcription of *IFNB1* gene and IFN-β expression, as also observed for HSV UL48 protein (30).

In the present study, significantly higher levels of serum IgA reactivity against recombinant HSV UL48 on ELISA were found in patients with BD, compared with healthy controls. In addition, patients with BD exhibited remarkable serum IgG reactivity against recombinant Hsc71, which was strongly associated with serum IgG reactivity against recombinant HSV UL48. In an effort to control antibody reactivity in various autoimmune diseases, several anti-viral treatments have been investigated (31). One such study demonstrated that oral acyclovir treatment could not effectively reduce the frequency and severity of BD-associated mucosal and ocular symptoms (32). Meanwhile, oral famciclovir treatment was shown to be effective in a BD-like mouse model (33). Since serum anti-HSV IgA and IgG antibodies present peaks with primary HSV infection and also show high levels during recurrent episodes, administering anti-viral agents with or without combined immune modulating treatments to reduce serum levels of anti-HSV antibodies may be a potential therapeutic option in BD (32–35).

Although we cannot describe the precise pathogenetic role of HSV UL48 or human Hsc71 in patients with BD, we suggest that HSV infection, especially massive exposure to the HSV UL48 protein, and impairment of human Hsc71 activity may be associated with the activation of autoreactive lymphocytes. However, further investigations are necessary to elucidate the roles of HSV UL48 and human Hsc71, as well as circulating IgA and IgG autoantibodies, against these proteins in patients with BD.

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The authors declare no conflicts of interest.

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